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The utility of exome sequencing for fetal pleural effusions

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Abstract

Objective: We sought to evaluate the performance of exome sequencing (ES) in determining an underlying genetic etiology for cases of fetal pleural effusions.

Study design: We examined a prospective cohort series of fetal pleural effusions visualized sonographically between 1 April 2016 and 31 August 2017. Fetal pleural effusions attributed to twin sharing, anemia, or structural anomalies were excluded, as were all cases with a genetic diagnosis established by karyotype or chromosomal microarray analysis. The remaining cases with pleural effusions of unclear etiology were offered ES. ES was performed by clinical sequencing and/or sequencing under the Baylor-Hopkins Center for Mendelian Genomics' (BHCMG) research platform. All cases were evaluated for novel genes or phenotypic expansion of disease-causing genes.

Results: ES was performed on six probands affected by pleural effusions. A pathogenic variant was identified in one case (16.7%). Four additional cases had variants of uncertain significance (VUS) in candidate genes of pathological interest. Neither clinical nor candidate genes were evident in the final case.

Conclusion: ES should be considered in the evaluation of prenatally detected idiopathic pleural effusions when other diagnostic workup for a genetic etiology has failed.

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CONFLICT OF INTEREST

Benjamin Solomon is employed by Gene Dx.

DATA AVAILABILITY STATEMENT

Data are available upon request.

1 | INTRODUCTION

Congenital pleural effusion is a condition affecting approximately 1:10 000 fetuses.¹ Progression is unpredictable as pleural effusions can spontaneously resolve or progress to fetal hydrops,² which can ultimately result in fetal or neonatal demise.³ Identification of the underlying condition is key to counseling and management. Currently, the evaluation involves a targeted ultrasonographic survey for structural anomalies, arrhythmias, and middle cerebral artery Doppler assessment to evaluate for fetal anemia.⁴ Amniocentesis or chorionic villus sampling is offered for chromosomal microarray, infectious evaluation, and potentially molecular gene panel testing, which includes RASopathies and metabolic conditions. Noonan syndrome is a known cause of fetal pleural effusions and has been diagnosed in similar cohorts by panel testing and whole exome sequencing.⁵ A diagnosis is unattainable in many cases, however, despite these laboratory evaluations. Thus, additional diagnostic tools are ultimately necessary to further elucidate the genetic cause of fetal pleural effusions and to provide appropriate counseling and management, which may include therapeutic interventions.

As one of a few conditions that may be successfully treated in utero,⁶ a careful and full evaluation of fetal pleural effusions is indicated in order to guide management. In the era of personalized medicine, pleural effusions are well positioned to benefit from targeted therapies based in part on the underlying diagnosis. Some examples of personalized in utero treatment include intrauterine blood transfusion for fetal anemia, digoxin for fetal arrhythmia,⁷ steroids for cystic pulmonary airway malformations,⁸ and pleural shunt placement or intrapleural injections for chylothorax.⁹

Recently, ES has identified additional candidate genes in cases with fetal pleural effusions. Reported cases involve rare single nucleotide variant changes in genes involved in biological pathways suggestive of causation, including *LZTR1*, *RIT1*, *KMT2D*,⁵ and *EPHB4*.¹⁰ In the current study, our primary goal was to identify the clinical yield of ES in fetuses with fetal pleural effusions of unclear etiology. Our secondary goal was to identify novel, candidate genes not yet confirmed as causative for fetal pleural effusions. We hypothesized that clinical prenatal ES is a useful diagnostic tool for the evaluation and management of fetal pleural effusions and to enhance our understanding of their pathogenesis and, thereby, improve and allow for individualized prognostic counseling.

2 | METHODS

We conducted a prospective cohort study of women who presented to the Johns Hopkins Hospital between 1 April 2016 and 31 August 2017 with fetal pleural effusions on perinatal ultrasound. The study population was defined as those who underwent ES after a negative sonogram for structural abnormalities, as well as a negative infectious, anemia (including red cell alloimmune), and chromosomal workup. The institutional review board at Johns Hopkins Hospital approved this study (NA_00045758). Research data are not shared.

Inclusion criteria required fetal pleural effusions (unilateral or bilateral), a normal karyotype, a negative infectious workup, and the absence of other structural anomalies aside from

hydrops fetalis. Exclusion criteria included multiple gestation, evidence of severe fetal anemia, any fetal karyotypic abnormality or chromosomal micro-deletion/duplication, and the presence of a structural fetal anomaly. All women received prenatal genetic counseling by certified genetic counselors, which encompassed pretest and posttest counseling. All study subjects were offered clinical or research ES, and all participants were appropriately consented. Participants were provided the option to opt out of obtaining research results. Proband and in some cases sibling DNA were extracted from amniocytes, neonatal cord blood, or neonatal blood. Parental DNA was extracted from peripheral blood or Oragene saliva kits. Clinical ES was performed through GeneDx, and research ES was performed through the Baylor-Hopkins Center for Mendelian Genomics (BHCMG). Rare variants were categorized as per the American College of Medical Genetics (ACMG) criteria as pathogenic, likely pathogenic, benign, likely benign, or variants of uncertain significance (VUS).¹¹ Common benign variants are not reported in this study. Family history and neonatal outcomes were collected by review of medical records.

The primary outcome was the percentage of clinically reportable pathogenic variants and VUS identified by ES. The secondary outcome was the identification of novel, candidate genes that have not yet been confirmed as causative for fetal pleural effusions.

2.1 | BHCMG sequencing method

DNA was extracted from saliva, blood, or amniotic fluid and submitted for ES. VerifyBAMID software was utilized to evaluate sample variant level quality and contamination. Variant Quality Score Recalibration (VSR) was utilized to assess the variant quality score and the single nucleotide variants indel and single variant calls. Whole exome sequencing was performed on exonic and flanking intron regions with the Agilent SureSelect HumanAllExonV5Clinical_S06588914 kit and paired end 125bp reads with the Illumina Hi-Seq 2500 platform. Variants with an internal or ExAC allele frequency of greater than 1% were removed, as were those with a depth of coverage <10.

2.2 | Interpretation

GeneDx sequencing results were interpreted by a clinical molecular geneticist, and data were included as per the clinical report issued to the patients. Variant call format (VCF) files of all patients, regardless of sequencing center, were then analyzed by the authors to identify pathogenic and additional, novel, candidate variants utilizing PhenoDB,¹² a publicly accessible analysis platform designed by the BHCMG. PhenoDB was utilized to analyze the cases by inheritance pattern, including autosomal dominant de novo or inherited variants, homozygous and compound heterozygous autosomal recessive variants, and X-linked variants. PhenoDB integrates information from several search platforms including OMIM, ClinVar, ExAC, and PolyPhen to allow for rapid interpretation of variants. Variants of interest were reviewed in the Integrative Genomics Viewer. Transmission disequilibrium tests were conducted from common variants (>5% mean allele frequency in 1000 genomes) to identify strong deviation from 0.5. Clinical history and family history, including consanguinity, were taken into consideration during interpretation. A clinical molecular geneticist verified each variant identified through research analysis.

3 | RESULTS

Of 33 cases with sonographically identified fetal pleural effusions (Figure 1), 23 (69.7%) were excluded because of twin-transfusion (five cases), twin anemia polycythemia sequence (one case), Rh disease (two cases), congenital diaphragmatic hernia (two cases), cystic pulmonary airway malformation (one case), fetal teratoma (two cases), monosomy X (six cases), trisomy 21 (two cases), trisomy 18 (one case) and terminal 7q36 deletion/7p22 duplication (one case).

The remaining 10 cases had a normal microarray and idiopathic pleural effusions of unclear etiology. Sequencing was not performed in four cases. Three of these cases declined all genetic testing: the first had a pregnancy termination, and two additional cases had late-onset pleural effusions with favorable neonatal outcomes. The final case had insufficient fetal sample retained for sequencing. Six cases underwent ES including four parent-proband trios and two parent-proband-affected sibling quads. The two cases with affected siblings also reported known consanguinity.

Of the cases that underwent ES, one pathogenic variant was identified (case 1, Table 1) for a total yield of 1/6 (16.7%). The pathogenic, paternally inherited variant was a stopgain variant (p.E111X) in *FOXC2*. This gene is causative for lymphedema distichiasis syndrome. The fetus was initially followed for a cystic hygroma that was visualized at 20 weeks gestation. A unilateral, right-sided pleural effusion developed at 35 weeks gestation. She underwent an induction of labor resulting in a vaginal delivery at 37 weeks 1 day gestation. Following identification of this gene, the father reported a history of lymphedema and corneal abrasions.

The five cases without a pathogenic variant were analyzed in PhenoDB. Candidate genes of possible pathological significance were identified in 4/5 of these cases. As in case 1, all four of the candidate genes are known to have pathogenic mutations that are expressed dominantly. Compound heterozygosity or homozygosity for variants in a candidate gene were encountered in three of the cases.

A heterozygous, maternally inherited variant in *EPHB4* was identified in case 2. Although suspected to be causative, it was categorized as a VUS by ACMG criteria on the basis of, in our case, a mildly affected maternal phenotype of intermittent lymphedema. In case 3, one proband variant in *RYR1*, a gene that has been associated with fetal hydrops, was inherited from each unaffected parent, thus resulting in compound heterozygous variants (p.P2567S and p.P816L) in the fetus.

Homozygous variants in *ACTN2* and *CRELD1* were identified in the affected fetuses and siblings of cases 4 and 5, respectively. These genes are known to cause autosomal dominant cardiomyopathy of the dilated or hypertrophic type, with or without left ventricular non-compaction (*ACTN2*) and partial atrioventricular septal defect with heterotaxy syndrome (*CRELD1*). The variants were characterized as VUS because of inheritance from unaffected parents who each had single heterozygous variants (Table 1). The result of double dominant homozygosity, however, has never been described for either of these genes.

4 | DISCUSSION

Trio or quad ES performed for cases with fetal pleural effusions of uncertain etiology identified a pathogenic variant in 16.7% of cases. Interestingly, none of the identified variants are in the Ras-MAP kinase pathway, which may or may not demonstrate multiple ultrasound findings prenatally in addition to pleural effusions or hydrops fetalis.¹³ Recent case reports of fetuses undergoing ES for pleural effusions have revealed a few genes such as *EPHB4*, which regulates angiogenesis. Such case reports have increased our recognition of the diverse genetic etiologies^{14,15} that may contribute to fetal pleural effusions. The present study has identified four novel candidate variants, through in-depth analysis of ES. Reporting such cases is important as part of the global collaborative effort to identify the role of genes in health and disease. Confirmation of these variants as the etiology of pleural effusions will require the identification of the same variants in future cases or reproduction of the phenotype in a murine or alternative animal model.

The one pathogenic variant identified in our cohort is a stopgain variant in forkhead box C2 (*FOXC2*) causing lymphedema distichiasis syndrome.¹⁶ The variant was paternally inherited and further implicated after sequencing when the father revealed his symptomatology. This couple also provided a history significant for multiple miscarriages. Variants in *FOXC2* have variable expressivity, and it is possible that variants in this gene were also responsible for fetal loss.¹⁷

We propose *EPHB4* to be a candidate gene on the basis of prior reported associations with fetal hydrops.^{9,18} Hydrops fetalis was reported in three independent families secondary to a genetic heterozygous variant in *EPHB4*. It was further confirmed by inactivation of *Ephb4* in a murine model resulting in lymphatic valve malformation.⁹ We suggest that our report, referring specifically to case 2, enhances the current literature with regard to the pathogenicity of *EPHB4* variants as a cause of pleural effusions as a manifestation of hydrops fetalis.

The skeletal muscle ryanodine receptor is encoded by the *RYR1* gene. Variable myopathic phenotypes¹⁹ as well as malignant hyperthermia²⁰ occur secondary to a variety of autosomal dominant missense mutations in heterozygous individuals. These are predominantly gain of function mutations in a heterozygous state that result in increased calcium efflux and a decrease in cellular calcium stores.²¹ Compound heterozygous and homozygous variants in *RYR1* have been previously reported in cases that resulted in hydrops.^{22,23} The *RYR1* receptor is important in many developmental pathways and thus is essential to fetal development. We believe our case number 3 in which compound heterozygous variants were found in a proband with a clinically related phenotype represents strong evidence for *RYR1* as an underlying causative gene.

In case 4, we identified a homozygous variant in *CRELD1*, a gene that is associated with autosomal dominant congenital heart disease due to haplo-insufficiency in the heterozygous state.²⁴ There is variable expressivity in the heterozygous autosomal dominant state, when only one pathogenic variant is present. It has been suggested that *CRELD1* modulates expression of a cardiac defect in cases with Down syndrome.²⁵ No homozygous cases have

previously been reported. The matricellular protein CRELD1 may function as a cell adhesive molecule with high expression in cardiac and skeletal muscle. It is possible that in case 4 the severe phenotype was due to the identified homozygous *CRELD1* variant. In this case the fetal pleural effusions that resulted in hydrops and demise could have been either due to cardiac dysfunction or a skeletal muscle defect.

Finally, the homozygous variant of *ACTN2* is suspected to be causative on the basis of the well-described autosomal dominant cardiac phenotype.²⁶ The rarity of the variant in ExAC (1.0×10^{-4}) and the known cardiomyopathy phenotype being consistent with our fetal presentation in case 5 makes *ACTN2* a good candidate gene. There are no previous reports to our knowledge as to the phenotype of the homozygous variant in this gene as was seen in case 5 of the present study.

Our study has several strengths, including a longitudinal cohort of all cases presenting with pleural effusions at a tertiary care center. We sequenced two cases with consanguinity and a prior affected sibling. This improved our a priori risk for identifying a homozygous gene that was causative for pleural effusions. These quads were ideal candidates for exome sequencing; however, the variants identified were limited by the lack of functional studies to confirm the impacts of these variants. We furthermore had the benefit of qualified clinical geneticists collaborating with molecular geneticists to perform in-depth analysis.

This study also has several limitations. Because of the rarity of cytogenetically normal fetal pleural effusions of unclear etiology, we were only able to include six cases that underwent ES. Although neonatal sequencing for congenital anomalies is standard of care, it is only recently that we are sequencing cases with fetal conditions, and specifically fetal lethal conditions. As such, there is a lack of comparative data to confirm or refute the proposed pathogenicity of the identified candidate genes. Our proposed candidate genes may or may not be confirmed in the future. We must also recognize the possibility that, because of a lack of known causative variants, we missed the true pathogenic variant in the causative gene during our analysis. It is also possible that this variant was not identifiable due the limitations of our exome sequencing platform, either in terms of depth of coverage or capture efficiency.

Despite these limitations, this study contributes plausible evidence for several genetic etiologies underlying fetal pleural effusions. At minimum, our data demonstrate a clinical ES utility of 16.7%, which is on par with the diagnostic yield of ES for other prenatally diagnosed anomalies.²⁷ We propose four candidate genes, which, if proven, will expand our understanding of the pathogenic mechanisms that result in fetal pleural effusions. As the field moves forward, these genes could be included on a cell-free DNA platform to improve early diagnosis. Our study highlights in particular the importance of ongoing clinical investigation through ES in cases with fetal pleural effusions to further delineate pathogenic variants in candidate genes.

5 | CONCLUSION

ES should be considered in the evaluation of prenatally detected idiopathic pleural effusions when other diagnostic workup is unrevealing as to the underlying etiology. Our study found ES to be informative in one of six such cases, and in addition, several genes have arisen as suitable etiologic candidates. In our study of pleural effusions, none of the identified variants were in the Ras-MAP kinase pathway. Future studies are needed to develop the most cost-effective diagnostic approach to isolated idiopathic fetal pleural effusion. There is certainly a role for ES in the research setting to further classify the pathogenicity of rare, candidate genes associated with fetal pleural effusions.

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What is already known about this topic?

- Genetic etiologies of fetal pleural effusions have been identified by karyotype, chromosomal microarray, and targeted gene testing for RASopathies.
- Case reports of single gene variants suggest that exome sequencing (ES) will identify additional variants that are causative.

What does this study add?

- ES for cases with fetal pleural effusions identified a pathogenic variant in 16.7% of cases. Four additional candidate genes were identified (67% of cases).
- Identification of the underlying genetic etiology can aid in prognostic counseling, as well as in prenatal and postnatal management and/or treatment options for fetal pleural effusions.
- ES is useful in delineating the underlying genetic spectrum of fetal pleural effusions.

FIGURE 1.
Bilateral fetal pleural effusions

TABLE 1

Single gene variants in cases with fetal pleural effusion

Case	Proband Phenotype	Gestational Age of Onset	Gene/Inheritance	Outcome	OMIM Condition (All Autosomal Dominant)	ExAC	PolyPhenACMG Classification	ACMG Classification
1	History of recurrent miscarriage, fetal cystic hygroma, right pleural effusion	25 wk 4 d	<i>FOXG2</i> , Stopgain p.E111X Paternal, affected	Alive	Lymphedema-distichiasis	–	–	Pathogenic
2	Pleural effusion, pericardial effusion, scalp edema	32 wk 5 d	<i>EPHB4</i> Missense p.V870G Maternal, mild lymphedema	Neonatal demise	Hydrops, varicose veins, atrial septal defect	–	1.0	VUS
3	Pleural effusions, scalp edema, ascites, fetal growth restriction	24 wk 2 d	<i>RYR1</i> Missense p.P816L maternal p.P2567S paternal Fetus compound heterozygous	Fetal demise	Central core disease, King-Denborough syndrome, Neuromuscular disease, congenital, with uniform type 1 fiber	3.3×10^{-5} 2.5×10^{-5}	0.99 0.02	VUS
4 ^a	History of consanguinity and prior affected, fetal cystic hygroma, pleural effusions, ascites	18 wk 4 d	<i>CRELD1</i> Missense p.R329C Maternal/paternal heterozygous Fetus and sibling	Fetal demise	Atrial-ventricular septal defect, susceptibility	0.0006	1.0	VUS
5 ^a	History of consanguinity and prior affected, fetal pleural effusions, pericardial effusion, ascites, supraventricular tachycardia	33 wk 5 d	<i>ACTN2</i> Missense p.T412M Maternal/paternal heterozygous Fetus and sibling homozygous	Neonatal demise	Dilated cardiomyopathy	0.0001	0.009	VUS
6	Pleural effusions, supraventricular tachycardia, resolved with digoxin and flecainide	30 wk 3 d	None	Alive	–	–	–	–

Note. All probands had a normal chromosomal microarray.

Abbreviations: ACMG, American College of Medical Genetics; ExAC, Exome Aggregation Consortium; OMIM, Online Mendelian Inheritance in Man; PolyPhen, Polymorphism Phenotyping (1 = damaging); VUS, variants of uncertain significance.

^aQuad testing. Proband, mother, father, affected sibling.